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New Records of *Asplenium varians* (Aspleniaceae) and Two New Hybrids in Japan

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Asplenium varians is widely distributed in the Sino-Himalayan region and in Africa, but was newly recorded in Japan, where it occurs at two localities approximately 50 km apart in Miyazaki Prefecture, Kyushu. Two interspecific hybrids involving *A. varians* (i.e., *A. anogrammoides* × *A. varians* and *A.* ×*tenuivarians*) were also growing at the periphery of the *A. varians* populations. We confirmed the identity of the hybrids by chloroplast *rbcL* sequences, chromosome numbers and morphological features.

Key words: *Asplenium*, hybrid, Japan, Miyazaki, new distribution

Asplenium varians Wall. ex Hook. & Grev. (Aspleniaceae) is widely distributed in Asia (China, Bhutan, India, Nepal and Vietnam) and Africa (Viane & Reichstein 2003), but has not been recorded in Japan (Iwatsuki *et al*. 1995, Lin & Viane 2013), except as misidentification of plants now known to be *A. tenuicaule* Hayata (e.g., Ohwi 1957, Kurata & Nakaike 1981, 1997).

In 2011, specimens of *Asplenium* collected by Mr. Nobuyuki Inoue in Miyakonojo-shi, Miyazaki Prefecture were tentatively identified as *Asplenium* ×*shigeru-kobayashii* Nakaike, nom. nud. [a sterile hybrid between *A. anogrammoides* H. Christ (= tetraploid *A. sarelii*[1]) and *A. incisum* Thunb.]. The identification was later questioned because the population was very large, unlike typical sterile hybrid taxa, and because one of the putative parents, *A. incisum*, was not found nearby. After confirming that the plants produced regular, normally shaped spores, we considered the plants to be a species previously unknown in Japan. After examining herbarium specimens and descriptions of related species in the literature, we concluded that in gross morphology they best fit the description of *A. varians* [type image available in Nakaike (1992)].

Still, however, the origin of these plants through hybridization had to be considered. *Asplenium varians* is tetraploid, with $n = 72$, as reported from India (Lin & Sleep 1989) and China (Wang *et al*. 2003), and hybrids between *A. varians* and related species in *Asplenium* series *Variantia* Ching & S. H. Wu have been corroborated by Viane & Reichstein (2003) and Wang *et al*. (2003) based on information obtained from cytology, spore observations, allozyme analyses and morphological features (Fig. 1). To determine the genomic formulae is important in placing these

[1] According to the taxonomic scheme by Lin & Viane (2013), Japanese plant formerly called "*Asplenium sarelii*" (tetraploid) should be *A. anogrammoides*. The true *A. sarelii* Hook. is diploid, and unknown to Japan.

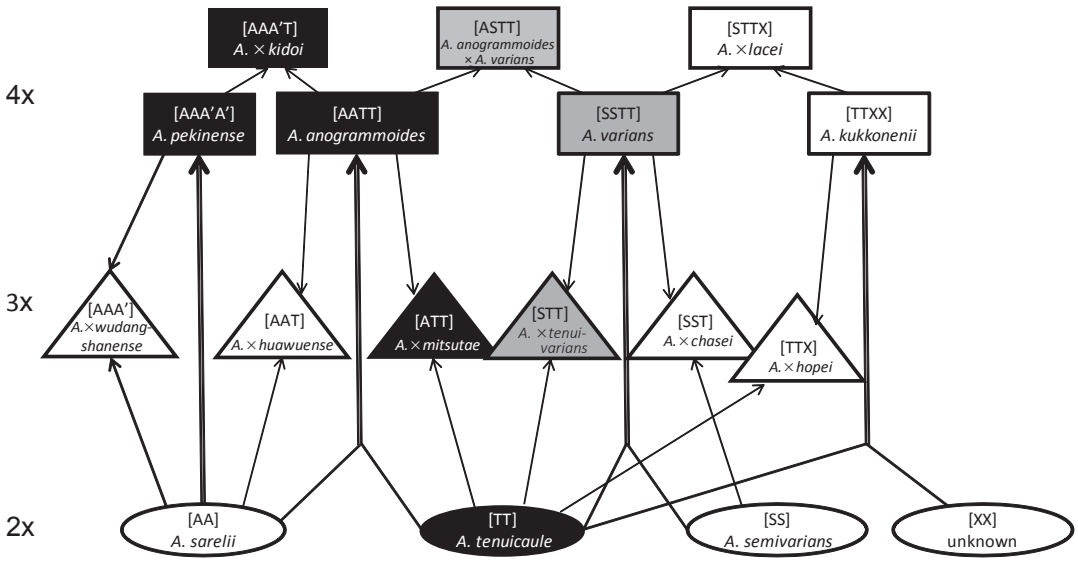

FIG. 1. Hypothesized speciation process of *Asplenium varians* and closely related species based on Lin & Sleep (1989), Lin & Viane (2013), Viane & Reichstein (2003) and Wang *et al*. (2003). Letters in square brackets indicate genomic formulae (A, A' = genome derived from *A. sarelii*; S = genome from *A. semivarians*; T = genome from *A. tenuicaule*; X = genome from unknown ancestral diploid species). Taxa with black background occur in Japan; gray background indicates taxa newly reported in Japan in this study; white background indicates taxa not in Japan.

plants on the existing reticulation map (Viane & Reichstein 2003, Wang *et al*. 2003).

Materials and Methods

Material and habitat

One population of *Asplenium varians* in Japan is extensive on a semi-shaded, moist, north-east-facing, concrete covered roadside cliff at Yamada, Miyakonojo-shi in southeastern Miyazaki Prefecture, at an altitude of approximately 200 m (Fig. 2A, C). The population, which extends for approximately 50 m, grows intermixed with *A. anogrammoides*, *A. tenuicaule*, *A. pekinense* Hance, and *A. yoshinagae* Makino. The surface of the concrete is almost completely covered by bryophytes (mostly *Hypopterygium fauriei* Besch.). The second locality, approximately 50 km from the first site at Idouchi, Nishimera-son in the western part of Miyazaki Prefecture, at an altitude of approximately 350 m (Fig. 2B, D), was discovered in 2012. The plants, on a concrete wall and concrete bridge, extended approximately 10 m, and were intermixed with *A. anogrammoides*, *A. tenuicaule*, *A. pekinense* and *A. yoshinagae*.

We collected five stocks from the first site and six stocks from the second site, including *Asplenium varians*, possible interspecific hybrids involving *A. varians*, and their potential parents. Following molecular analysis, we compared our results from these collections with analyses from known *A. varians*, including a sample of *A. varians* from Pakistan (AE3273, a cultivated collection at the Tsukuba Botanical Garden introduced in 1994[2]) as well as the *rbcL* sequence of *A. varians* based on stocks from China deposited in GenBank (Table 1). Extracted DNA and voucher

[2] According to Viane & Reichstein (2003), *A. varians* is unknown in Pakistan, but our material from Pakistan clearly shows the epidermal cell structure of *A. varians* (simple sinuous), not thickened or pseudomamillate as in *A. kukkonenii*, the species most often confused with *A. varians* (Nakato & Ebihara, unpublished).

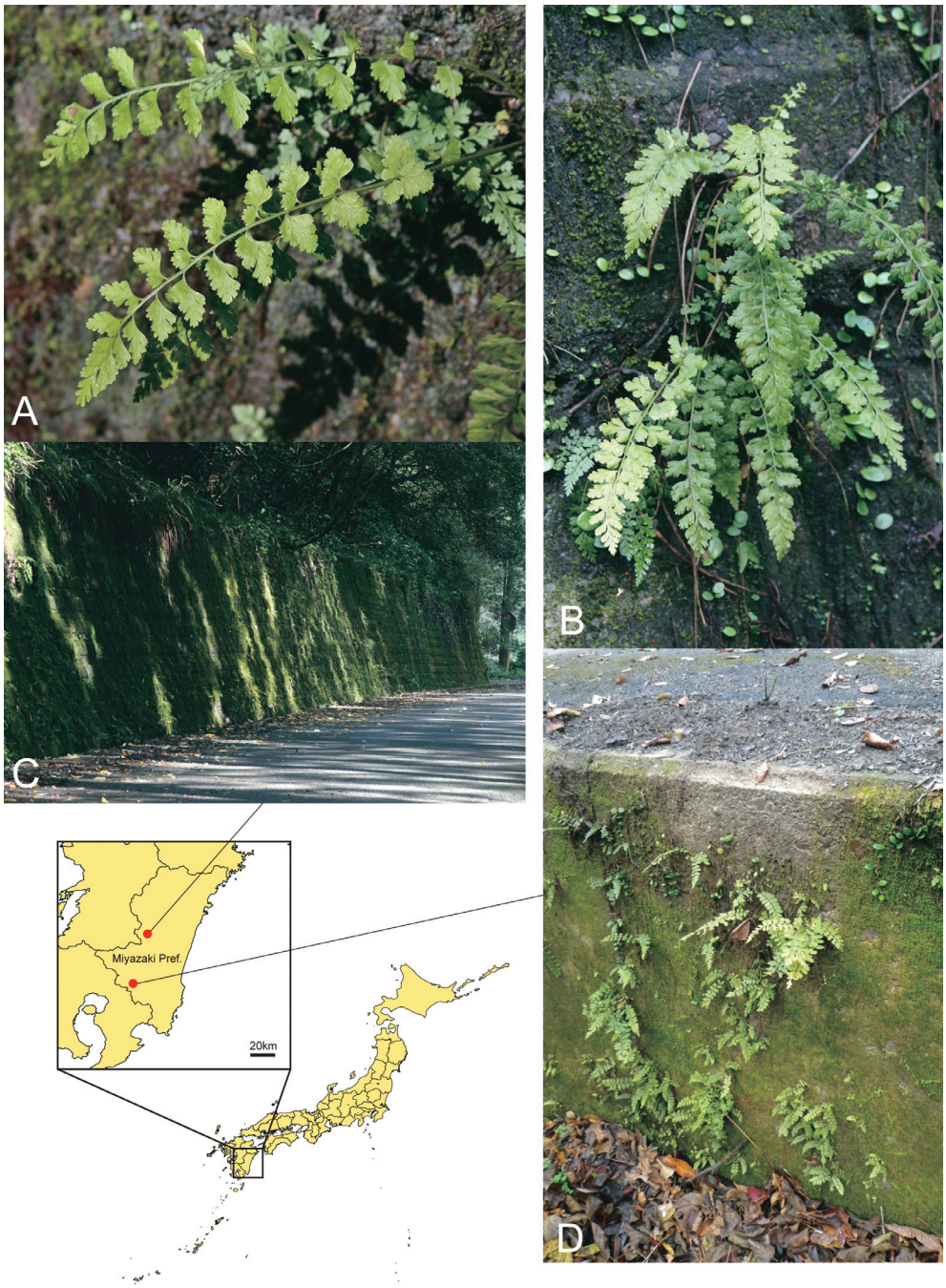

FIG. 2. Habitat of *Asplenium varians* in Japan (A, C: Miyakonojo-shi, Miyazaki Pref.; B, D: Nishimera-son, Miyazaki Pref.). (photographs by T. Minamitani).

specimens have been deposited both at the Center for Molecular Biodiversity Research and the herbarium (TNS) of the National Museum of Nature and Science, Japan.

Chromosome, spore, and epidermis observations

Root tips for mitotic chromosome counts were pretreated with 2 mM 8-hydroxyquinoline solution for 4 h, fixed in Carnoy's solution for 1–24 h,

TABLE 1. Materials examined in this study.

Species	Sample No.	Voucher (TNS-VS)	*rbcL* (GenBank accession)	Spore	Chromosome number	Locality	Reference
A. varians	*AE3206*	1176798	AB853875	normal	2*n* = ca.144 (4*x*)	Japan: Miyazaki Pref., Miyakonojo-shi, Yamada	
	AE3227	1176819	AB853876	normal	2*n* = 144 (4*x*)	Japan: Miyazaki Pref., Koyu-gun, Nishimera-son, Kamimera, Idouchi	
	AE3273	1181121	AB853877	-	-	Pakistan: Azad Kashmir, Chikkar (cultivated in Tsukuba Botanical Garden)	
	-	-	AY300147	-	-	China	Schneider *et al*. (2004)
	-	-	AY545478	-	-	China: Yunnan	Li & Lu (2006)
A. anogrammoides	*AE3049*	1170794	AB853878	-	-	Japan: Miyazaki Pref., Koyu-gun, Nishimera-son, Kamimera, Idouchi	
	AE3225	1176817	AB853879	normal	2*n* = 144 (4*x*)	same as above	
	-	769194	AB574873	-	-	Japan: Mie Pref.	Ebihara *et al*. (2010) *as A. sarelii*
A. anogrammoides × *A. varians*	*AE3209*	1176801	AB853880	abortive	2*n* = ca.144 (4*x*)	Japan: Miyazaki Pref., Miyakonojo-shi, Yamada	
	AE3210	1176802	AB853881	abortive	2*n* = 144 (4*x*)	same as above	
	AE3211	1176803	AB853882	abortive	2*n* = 144 (4*x*)	same as above	
	AE3212	1176804	AB853883	abortive	2*n* = ca.144, 36II + 72I (4*x*)	same as above	
A. tenuicaule	*AE3232*	1176824	AB853884	normal	2*n* = 72 (2*x*)	Japan: Miyazaki Pref., Koyu-gun, Nishimera-son, Kamimera, Idouchi	
	AE3051	1170796	AB853885	-	-	same as above	
	-	765223	AB574878	-	-	Japan: Yamagata Pref.	Ebihara *et al*. (2010)
A. ×*tenuivarians*	*AE3048*	1170793	AB853886	abortive	-	Japan: Miyazaki Pref., Koyu-gun, Nishimera-son, Kamimera, Idouchi	
Related taxa and outgroups							
A. capilipes		766625	AB574854		-	Japan: Kochi Pref.	Ebihara *et al*. (2010)
A. pekinense		762770	AB574866		-	Japan: Miyazaki Pref.	Ebihara *et al*. (2010)
A. pekinense		-	AY545479		-	China	Li & Lu (2006)
A. lushanense		-	AY545481		-	China	Li & Lu (2006)
A. trichomanes		-	EF463157		-	U.S.A.	Schuettpelz & Pryer (2007)
A. aegaeum		-	AY300103		-	Greek	Schneider *et al*. (2005)
A. marinum		-	AF240647		-	U.K.	Schneider *et al*. (2005)

macerated in 1 N HCl at 60°C for 1 min, and then squashed in aceto-orcein. For meiotic chromosome observations, young fertile fronds were fixed in Carnoy's solution for 12–24 h and the sporangia then squashed in aceto-orcein. Mature spores were embedded in Bioleit (Ohken Co., Tokyo, Japan), and their fertility was estimated by shape and size. Epidermal cell wall structure, useful for distinguishing some species in the *Asplenium varians* complex [*e.g.* simply sinuate (not mamillately thickened) walls in *A. varians* versus mamillate or slightly thickened walls in *A. kukkonenii* Viane & Reichst. (Viane & Reichstein 2003)], was observed in a sample preserved in ethanol (AE3206).

Molecular analysis

The plastid *rbcL* region (1190 bp) was used to infer maternal lineages. Methods for DNA extraction, amplification, and sequencing followed those in a previous study (Ebihara *et al*. 2010). The sequences of *Asplenium aegaeum* Lovis, Reichst. & Greuter and *A. marinum* L. from GenBank were used as definite outgroups based on

the topology obtained by Schneider *et al*. (2004). Maximum parsimony analysis was performed using MEGA 5.03 (Tamura *et al*. 2011) with the tree-bisection-regrafting search method, in which the initial trees were obtained by randomly adding the sequences (100 replicates). MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) was used to calculate the posterior probability of each node for Bayesian inference. The first quarter of 10 million generations was discarded as a burn-in period.

Results

Spore observations

Among the nine specimens examined, the spores of AE3048, AE3209, AE3210, AE3211, and AE3212 were abortive or irregularly shaped; those of the remaining samples were normal and bilateral (Fig. 3, Table 1). Frond morphology of AE3048 was intermediate between *Asplenium tenuicaule* and *A. varians*. The morphology of AE3209 through AE3212 was intermediate between *A. anogrammoides* and *A. varians*.

Chromosome observations

Mitotic chromosome numbers of $2n = 144$ and $2n$ = approximately 144 were counted in seven samples from the two sites (Yamada, Miyakonojo-shi and Kamimera, Nishimera-son, Miyazaki Pref.). One sample from the latter site (AE3232, *Asplenium tenuicaule*) was $2n = 72$ (Fig. 4, Table 1). We also observed a meiotic chromosome configuration of 36II + 72I in a possible hybrid (AE3212).

Epidermal cell observations

Simple sinuous epidermal cell walls were observed on the abaxial surface of sample AE3206 (Fig. 5).

Molecular analysis

Three distinct clades, corresponding to *Asplenium tenuicaule* ('clade T'), *A. varians* ('clade S') and (*A. anogrammoides* + *A. pekinense*) ('clade A') were recognized in the tree obtained (Fig. 6). All sequences of *A. varians* from multiple localities distant from each other (two localities in Miyazaki Prefecture, Japan, China, and Pakistan) matched completely. In the suspected hybrids between *A. anogrammoides* and *A. varians* (see the discussion below), two of the four samples had the same *rbcL* sequence as Clade S; the two remaining samples were included in clade A. The sequence of the possible hybrid between *A. tenuicaule* and *A. varians* (see the discussion below) matched that of *A. tenuicaule*.

Discussion

Asplenium varians *in Japan*

Although no differences were found between the *rbcL* sequences of *Asplenium varians* in Japan and *A. varians* from China and Pakistan, this may not indicate conspecificity, considering the reticulate relationships already known within this group (Fig. 1, modified and expanded based on Lin & Sleep (1989), Viane & Reichstein 2003, Wang *et al*. 2003). We also confirmed that *A. varians* in Japan is a tetraploid ($2n = 144$) with normal spores with simple sinuous cell walls of the abaxial epidermis. This combination of characteristics is known only in *A. varians* (Viane & Reichstein 2003), thereby supporting the presence of *A. varians* in Japan.

Asplenium varians is hypothesized to be an allotetraploid derived from hybridization between *A. semivarians* and *A. tenuicaule* (Viane & Reichstein 2003). The speciation hypothesis itself, mostly inferred from chromosome number and behavior at meiosis in artificially crossed individuals, is awaiting verification utilizing biparentally inherited nuclear DNA markers.

Although the habitats at the two localities of *Asplenium varians* in Japan are man-made structures along paved roads, the plants likely were distributed naturally. Such man-made habitats are often preferred by some species of *Asplenium*, including *A. anogrammoides*, *A. incisum*, *A. pekinense*, and *A. tenuicaule* (A. Ebihara, personal observation). *Asplenium varians* grows in similar habitats in China (Lin & Viane 2013).

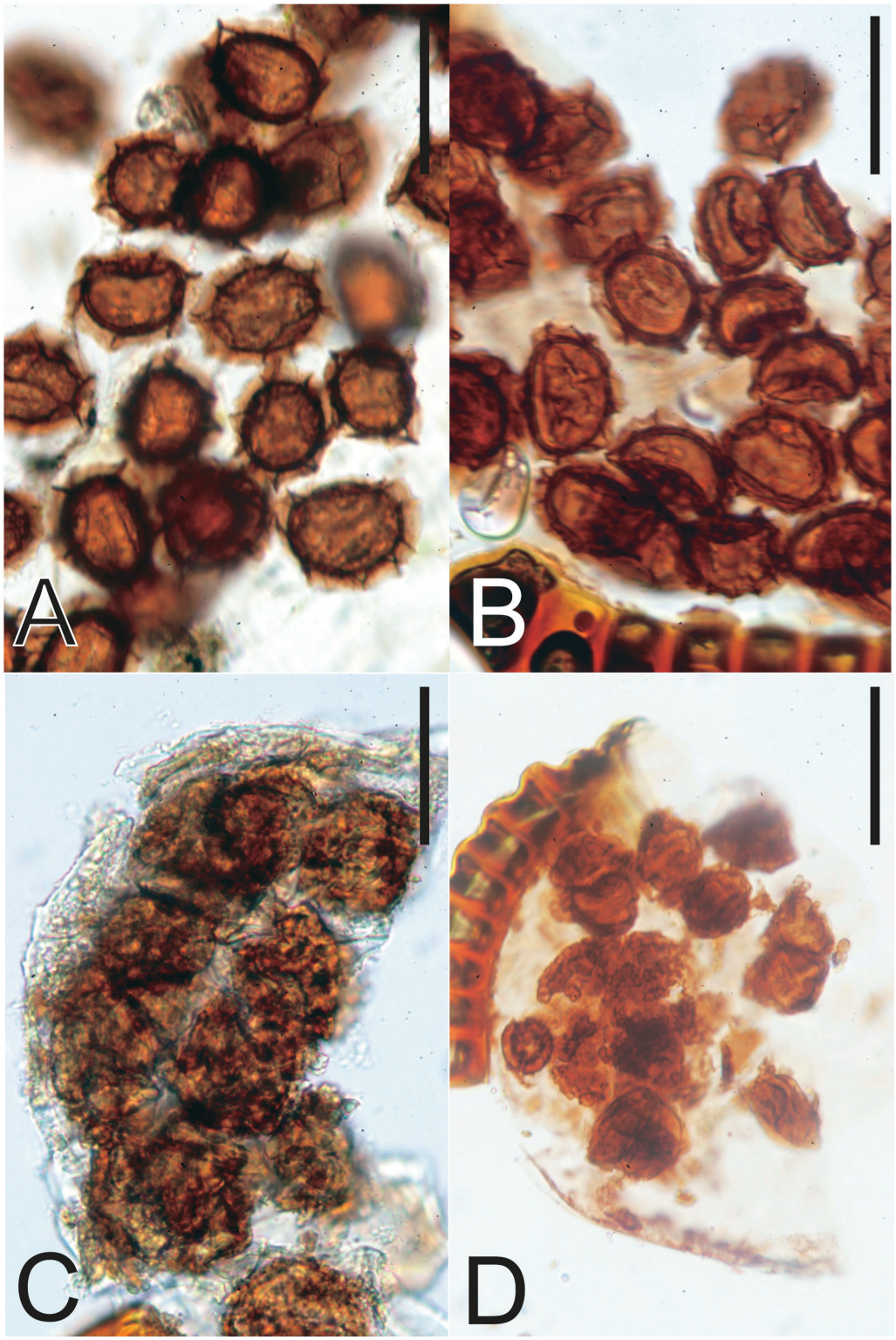

FIG. 3. Spores of *Asplenium varians* and related species. A: *A. varians* (normal, AE3227); B: *A. anogrammoides* (normal, AE3225), C: *A. anogrammoides* × *A. varians* (irregular, AE3210) and D: *A.* ×*tenuivarians* (irregular, AE3048). Scales: 50 μm.

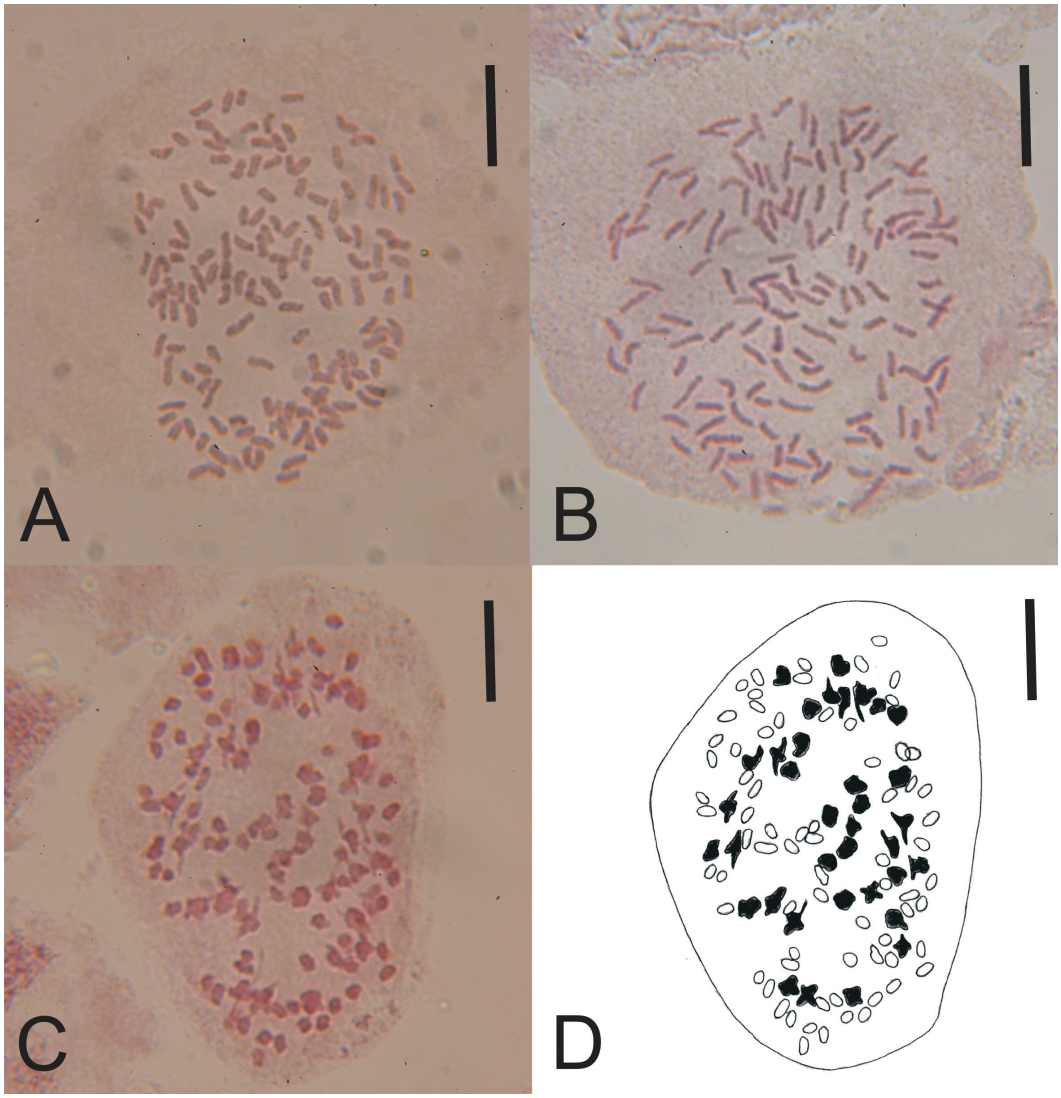

FIG. 4. Chromosomes of *Asplenium varians* and *A. anogrammoides* × *A. varians*. A: mitotic metaphase of *A. varians* (2*n* = 144, AE3227); B: mitotic metaphase of *A. anogrammoides* × *A. varians* (2*n* = 144, AE3211); C: first meiotic metaphase of *A. anogrammoides* × *A. varians* (AE3212). D: Explanatory illustration of C, showing 36 bivalents (black) and 72 univalents (white). Scales: 10 μm.

Interspecific hybrids

Four samples (AE3209 through AE3212) were determined to be interspecific hybrids between *Asplenium anogrammoides* and *A. varians* based on the combination of results from our study, namely plastid *rbcL* sequences matched one of the putative parents, abortive spores, tetraploid chromosome number of 2*n* = 144 or approximately 144, partly unpaired meiotic chromosomes and intermediate frond morphology. The fronds are similar to *A. varians*, but the rarely overlapping (more spaced) segments and more or less thicker lamina than in *A. varians* suggest involvement of *A. anogrammoides*. As judged from the chloroplast sequences, *A. varians* was the maternal parent in two samples (AE3209 and AE3210) and the paternal parent in the other two (AE3211 and AE3212).

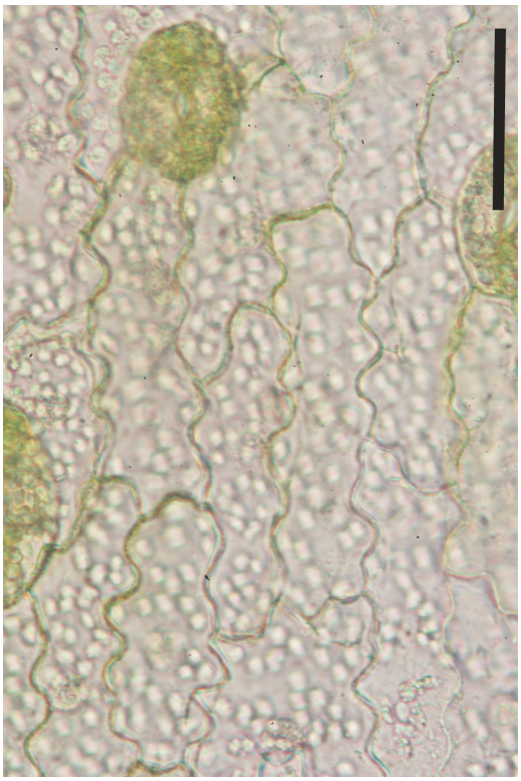

FIG. 5. Structure of epidermal cell on abaxial surface of frond of *Asplenium varians* (AE3206). Scale: 50 μm.

The 36II + 72I meiotic chromosome configuration is well explained by the speciation hypothesis for *Asplenium varians* and closely related species (Fig. 1). Viane & Reichstein (2003) and Wang *et al*. (2003) assumed the involvement of several diploid progenitors, including *A. sarelii* (genomic formula: AA^3), *A. semivarians* Viane & Reichst. (genomic formula: SS) and *A. tenuicaule* (genomic formula: TT). They suggested that *A. varians* is an allotetraploid (genomic formula: SSTT) that originated from hybridization between *A. semivarians* and *A. tenuicaule*, and that *A. anogrammoides* [= *A. wudangense* sensu Wang *et al*. (2003)] is an allotetraploid (AATT) that originated from hybridization between *A. sarelii* and *A. tenuicaule* (Lin & Viane 2013). If these hypotheses are accepted, the hybrid between *A. anogrammoides* (AATT) and *A. varians* (SSTT) is a tetraploid with a genomic formula SATT, and the 36 bivalent chromosomes are probably derived from *A. tenuicaule*. A hybrid of this combination was once recorded by Khullar (1994) in India but later regarded as a misidentification of *A.* ×*lacei* Reichst. & Viane by Fraser-Jenkins (2008) on the basis of the absence of *A. anogrammoides* in India. Therefore, this study is probably the first reliable record of the hybrid between *A. anogrammoides* and *A. varians*.

We observed abortive spores and obtained an *rbcL* sequence exactly the same as in *A. tenuicaule* in a plant morphologically intermediate between *Asplenium varians* and *A. tenuicaule* (AE3048; with longer basal pinnae than typical *A. varians*). Although cytological data are unavailable, it is highly probable that this individual is an interspecific hybrid between *A. tenuicaule* and *A. varians*. A hybrid of this combination, *A.* × *tenuivarians*, nom. nud., is known from China (Wang *et al*. 2003).

Taxonomic treatment

Asplenium varians Wall. ex Hook. & Grev., Icon. Filic., t. 172. 1830. —Fig. 7

Rhizome ascending to suberect, covered by dark brown clathrate scales. Stipes 1–3 mm in diameter, green, 4–15 cm long, abaxial surface of lower part more or less purplish; scales dark brown, entire, 2–4(–5) × 0.2–1 mm in basalmost part , caducous in apical part; lamina bipinnate, narrowly-elliptic to lanceolate, apex acute, more or less dimorphic, fertile lamina 10–20 × 4–8 cm, sterile lamina 3–10 × 1–2 cm, abaxial surface pale green, adaxial surface yellowish green to bright green; both surfaces of rachis green, adaxial surface of rachis grooved; pinnae 10–12 pairs at intervals of approximately 1 cm, stalked, triangular-ovate to triangular-lanceolate, 1.5–4.0 × 1.5–1.8 cm, apex acuminate to obtuse; basal pinnae shorter than middle pinnae, approximately half as long as longest pinnae; adaxial surface of rachis of pinnae shallowly grooved; basal acroscopic pinnules larger than basiscopic ones, ovate

[3] Wang *et al*. (2003) originally used the letter S for the genome of *A. sarelii*, but we use the letter A to avoid duplication.

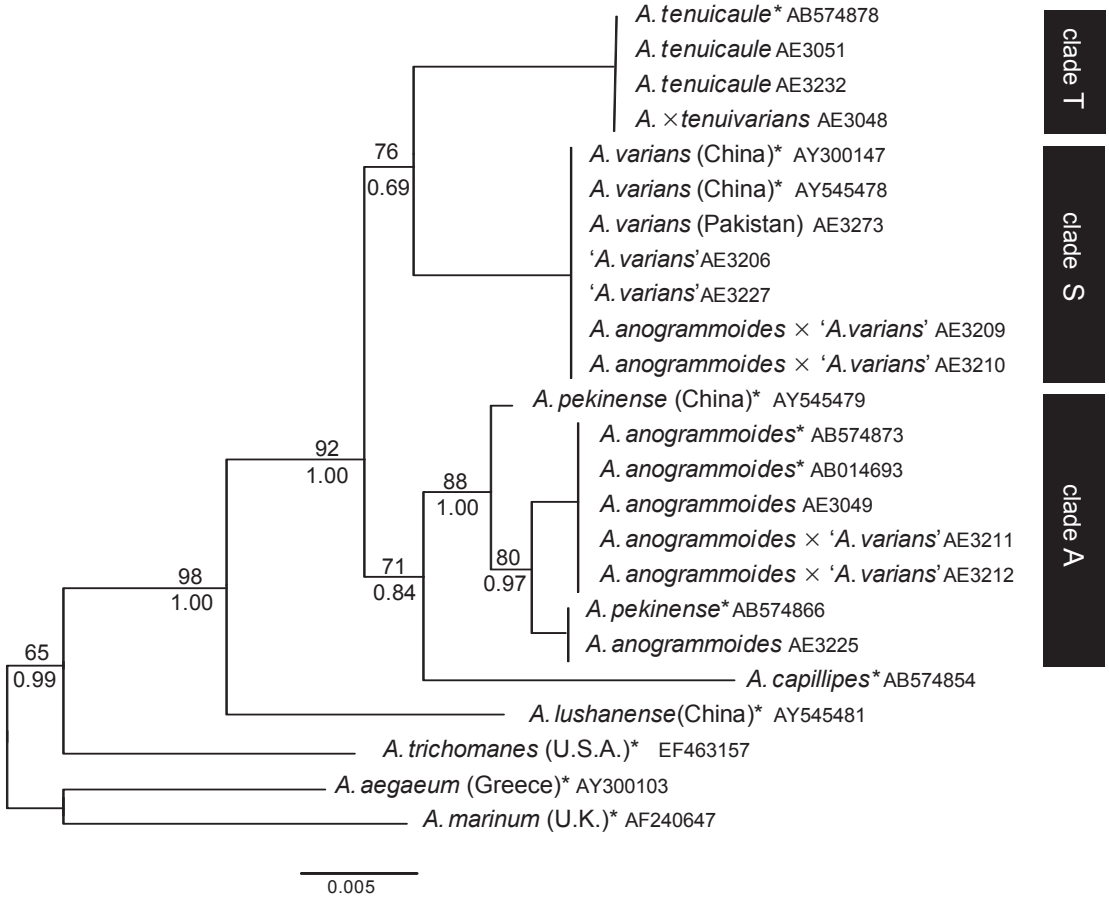

FIG. 6. A *rbcL* tree inferred by Bayesian method. Support values: Bootstrap test (1000 times) in maximum parsimony analysis (above branches) and posterior probabilities of Bayesian inference (below branches). Sequences with asterisks are from GenBank. Materials were collected in Japan or otherwise indicated.

to round, ca. 4–6 × 4–6 mm, remaining pinnules obovate, margin crenate to serrate. Sori 2–4 per pinnule, narrowly elliptic, 2–4 × 0.5 mm; indusia not inrolled, margin subentire or slightly dentate. Spores bilateral; perispore present. Chromosome number $2n = 144$.

The description is based chiefly on Japanese plants, but matches the description in Lin & Viane (2013).

Distribution. Japan (Kyushu), Bhutan, China, India, Nepal, Pakistan, Vietnam; Africa

Japanese name. Inoue-toranoo (named for Mr. Nobuyuki Inoue, who first discovered *Asplenium varians* in Japan).

Note. The circumscription of *Asplenium varians* follows Lin & Viane (2013); we simply added Japan and Pakistan to the range of distribution. The name *A. varians* is controversial, since it has been revealed that the type specimen of *Asplenium laciniatum* D. Don (Prodr. Fl. Nepal, 8, 1825) comprising 'a depauperate single frond with a few spores' is assignable to a taxon closely related to *A. varians* (Lin & Viane 2013). Even though *A. laciniatum* may be the earlier name for this taxon, we prefer to use *A. varians*, following Lin & Viane (2013).

Asplenium anogrammoides H. Christ × **A. varians** Wall. ex Hook. & Grev. —Fig. 8A, C

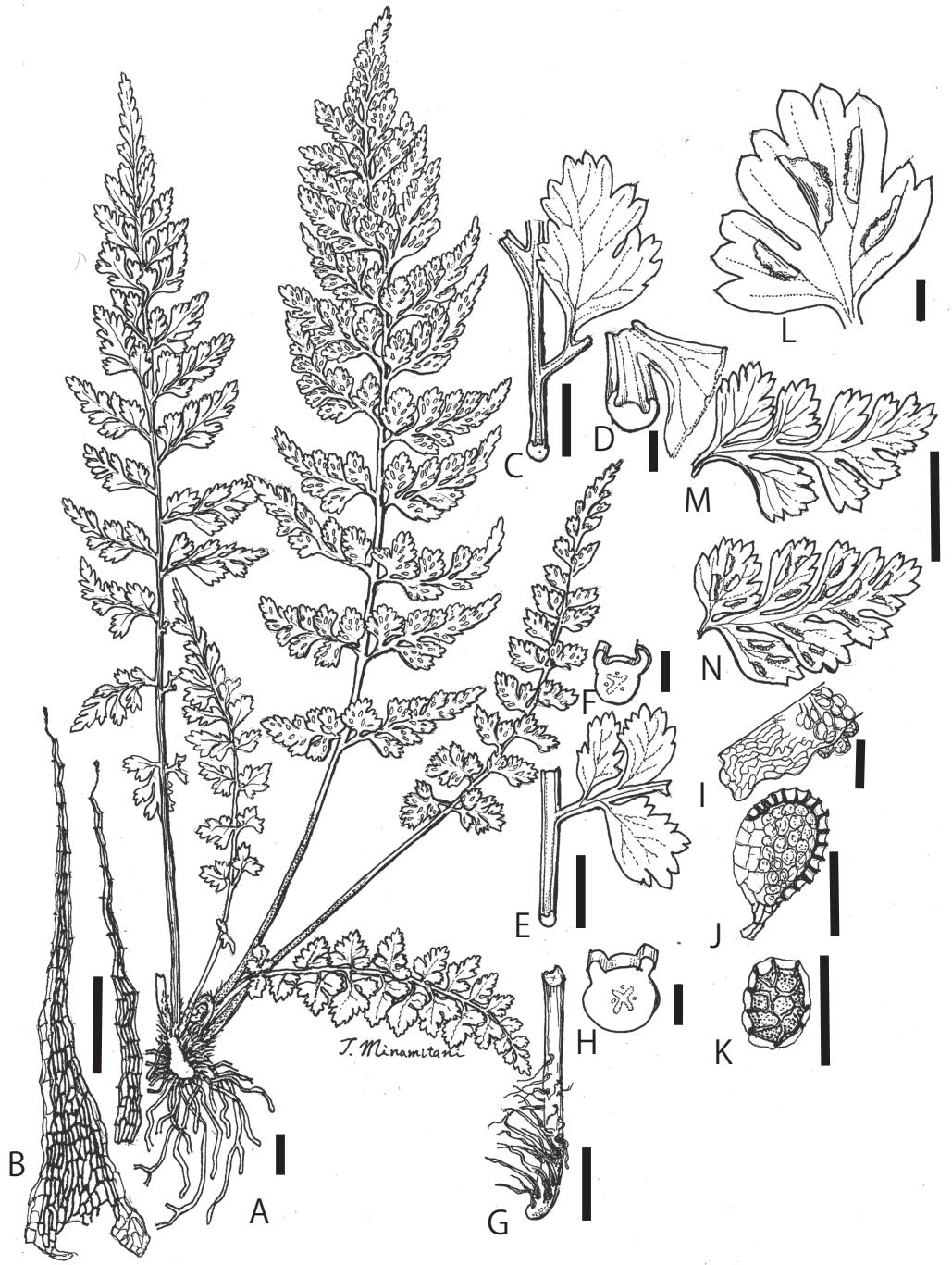

FIG. 7. *Asplenium varians* from Japan [T. Minamitani *s.n.* (TNS), Yamada, Miyakonojo-shi, Miyazaki Prefecture]. A: Whole plant; B: scales on lower stipe; C: adaxial surface of upper rachis; D: cross section of upper rachis; E: adaxial surface of lower rachis; F: cross section of lower rachis; G: base of stipe; H: cross section of stipe; I: indusium; J: sporangium; K: spore; L: basal acroscopic pinnule of basal pinna; M: adaxial surface of pinna; N: abaxial surface of pinna. Scales: 1 cm for A, M, and N; 1 mm for B, D, F, H, L, M, and N; 5 mm for C, E, and G; 500 μm for I; 250 μm for J; 50 μm for K. (line drawings by T. Minamitani).

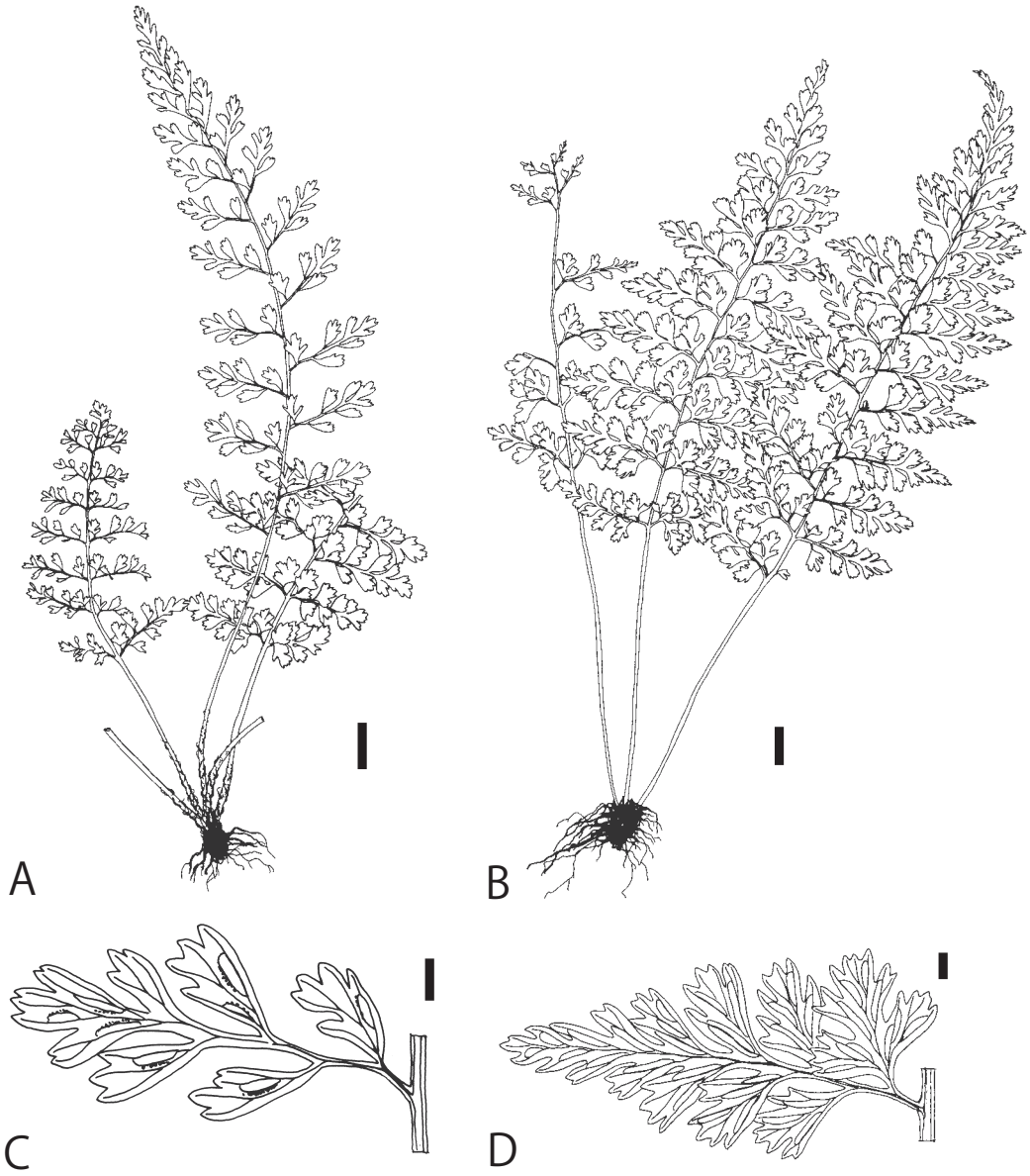

FIG. 8. A, C: *Asplenium anogrammoides* × *A. varians* (AE3211; A: whole plant; C: lower pinna); B, D: *A.* ×*tenuivarians* (AE3048; B: whole plant; D: lower pinna). Scales: 1 cm for A, B; 2 mm for C, D. (line drawings by A. Sasaki).

Similar to *Asplenium varians*, but pinnules more spaced; basal pinnae nearly as long as or slightly shorter than second lowest pinnae; lamina more or less thicker than *A. varians*. Spores abortive, tetraploid, 2*n* = 144.

Distribution. Japan (Kyushu)

Japanese name. Koba-no-inoue-toranoo

Asplenium ×tenuivarians Z. R. Wang ex Ebihara, **hybr. nov.** —Fig. 8B, D

— *A.* ×*tenuivarians* Z. R. Wang, nom. nud., Acta Bot. Sin. 45: 11, 2003.

Similar to *Asplenium varians*, but lamina subdeltoid;

basal pinnae nearly as long as second lowest pinnae; spores abortive.

Typus. JAPAN. Miyazaki Prefecture, Koyu-gun, Nishimera-son, Idouchi, alt. ca. 350 m. June 30, 2012, *T. Minamitani s.n. [AE3048]* (holo- TNS[VS-1170793]).

Asplenium ×*tenuivarians* is a sterile hybrid between *Asplenium tenuicaule* Hayata and *A. varians* Wall. ex Hook. & Grev.

Rhizome ascending to suberect, covered with dark brown, clathrate scales. Stipe to 10 cm long, 1–3 mm in diameter, green, abaxial surface of lower part more or less purplish; scales dark brown, entire, caducous, basally 4 × 1 mm, apically slender; lamina bipinnate, subdeltoid, to 16 × 6 cm, apex acute, sterile laminae 3–10 × 1–2 cm, abaxial surface pale green, adaxial surface yellowish green or bright green, both surfaces of rachis green, adaxially surface of rachis grooved; pinnae stalked, around 15 pairs at intervals of approximately 1 cm, triangular-ovate, to 3.5 × 2.0 cm, apex acute to obtuse, basal pinnae longest; adaxial surface of rachis of pinnae shallowly grooved; basal acroscopic pinnules largest, ovate to round, to 10 × 8 mm, remaining pinnules obovate, margin crenate to serrate. Sori 3–8 per pinnule, narrowly elliptic, 2–4 × 0.5 mm; indusia not inrolled, margin subentire or slightly dentate. Spores abortive.

Distribution. Japan (Kyushu). Probably also in China (Yunnan), but we have not examined the specimen cited by Wang *et al*. (2003). Lin & Viane (2013) noted "In places where *Asplenium tenuicaule* and *A. varians* grow together, their sterile hybrid is not uncommon [in China]".

Japanese name. Inoue-iwa-toranoo.

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